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# Magnesium sulphate induces preconditioning in preterm rodent models of cerebral hypoxia-ischemia

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## ABSTRACT

**Background:** Brain injury in preterm infants represents a substantial clinical problem associated with development of motor impairment, cognitive deficits and psychiatric problems. According to clinical studies, magnesium sulphate (MgSO<sub>4</sub>) given to women in preterm labor reduces the risk of cerebral palsy in the offspring but the mechanisms behind its neuroprotective effects are still unclear. Our aim was to explore whether MgSO<sub>4</sub> induces tolerance (preconditioning) in the preterm rodent brain. For this purpose we established a model of perinatal hypoxia-ischemia (HI) in postnatal day 4 rats and also applied a recently developed postnatal day 5 mouse model of perinatal brain injury.

**Methods:** Postnatal day 4 Wistar rats were exposed to unilateral carotid artery ligation followed by 60, 70 or 80 min of hypoxia (8% O<sub>2</sub>). On postnatal day 11, brains were collected and macroscopically visible damage as well as white and grey matter injury was examined using immunohistochemical staining. Once the model had been established, a possible preconditioning protection induced by a bolus MgSO<sub>4</sub> injection prior to 80 min HI was examined 7 days after the insult. Next, a MgSO<sub>4</sub> bolus was injected in C57Bl6 mice on PND 4 followed by exposure to unilateral carotid artery ligation and hypoxia, (10% O<sub>2</sub>) for 70 min on PND 5. Brains were collected 7 days after the insult and examined with immunohistochemistry for grey and white matter injury.

**Results:** In rats, a 60 min period of hypoxia resulted in very few animals with brain injury and although 70 min of hypoxia resulted in a higher percentage of injured animals, the brains were marginally damaged. An 80 min exposure of hypoxia caused cortical tissue damage combined with hippocampal atrophy and neuronal loss in the C3 hippocampal layer.

In the rat model, MgSO<sub>4</sub> (1.1 mg/g administered *i.p.* 24 h prior to the induction of HI, resulting in a transient serum Mg<sup>2+</sup> concentration elevation to 4.1 ± 0.2 mmol/l at 3 h post *i.p.* injection) reduced brain injury by 74% in grey matter and 64% in white matter.

In the mouse model, MgSO<sub>4</sub> (0.92 mg/g) *i.p.* injection given 24 h prior to the HI insult resulted in a Mg<sup>2+</sup> serum concentration increase reaching 2.7 ± 0.3 mmol/l at 3 h post injection, which conferred a 40% reduction in grey matter injury.

**Conclusions:** We have established a postnatal day 4 rat model of HI for the study of preterm brain injury. MgSO<sub>4</sub> provides a marked preconditioning protection both in postnatal day 4 rats and in postnatal day 5 mice.

## 1. Introduction

Brain injury in preterm infants is a major clinical problem causing

mortality and neurological morbidity (Ferriero 2004; Hagberg et al., 2015). The risk of neurodevelopmental impairment is inversely proportional to gestational age at birth (Kurinczuk et al., 2010).

**Abbreviations:** ADHD, attention deficit hyperactive disorder; CP, cerebral palsy; HI, hypoxia-ischemia; *i.p.*, intraperitoneal; IVH, intraventricular hemorrhage; MAP-2, microtubule-associated protein-2; MBP, myelin basic protein; Mg<sup>2+</sup>, magnesium; MgSO<sub>4</sub>, magnesium sulphate; PC, preconditioning; PND, post-natal day; preOLs, pre-myelinated oligodendrocytes; PVL, periventricular leukomalacia

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Approximately 30% of infants born preterm (< 28 weeks of gestation) will suffer from CP, cognitive impairment or behavioral deficits including autism spectrum disorders and ADHD (Brown et al., 2009; Marlow et al., 2005; Volpe 2009a). Therefore there is an imperative need to develop innovative treatment and preventive strategies.

Preterm brain injury in the human infant (encephalopathy of prematurity) includes both overt brain injury often combined with periventricular/intraventricular hemorrhage (PVH/IVH) preferentially in white matter (sometimes described as periventricular leukomalacia, PVL) and impairment of brain development affecting both grey and white matter as well as neuronal connectivity (Ball et al., 2015; Bataille et al., 2017; Inder et al., 1999a; Kidokoro et al., 2014; Pierson et al., 2007; Volpe 2009b). In the white matter, pre-myelinated oligodendrocytes (preOLs) are affected (causing the inability of these cells to differentiate into mature oligodendrocytes) leading to cerebral hypomyelination (Back and Miller 2014; Ferriero 2004). Due to the large quantity of preOLs in the periventricular white matter between gestational weeks 24–32, the neonatal human brain is highly susceptible to hypoxia-ischemia (HI) during this period of development. In rodents, the amount of preOLs is at its peak between post-natal day (PND) 2 and PND 5 (Back et al., 2001) and at PND 6/7 the majority of preOLs are already differentiating into mature oligodendrocytes (OLs) allowing early myelination to take place (Craig et al., 2003).

The etiology behind brain lesions in preterm infants is multifactorial and partly unknown but most likely includes infection/inflammation, hyperoxia, hypoxia, ischemia, excitotoxicity and injury secondary to IVH/PVH (Favrais et al., 2011; Hagberg et al., 2002; Volpe 2009b). In order to model HI in animals corresponding to the human at preterm, the Rice-Vannucci model has been adapted to younger animals including PND 5 (Albertsson et al., 2014), PND 6 (Shen et al., 2010) and PND 7 mice as well as PND 3 rats (Sizonenko et al., 2003).

Antenatal magnesium sulphate (MgSO<sub>4</sub>) has been shown to exert neuroprotective effects and according to clinical cohort studies, the offspring to mothers treated with MgSO<sub>4</sub> antenatally (administered for prevention of preterm labor or as seizure prophylaxis in preeclampsia) had a lower occurrence of both CP (Nelson and Grether 1995) and intraventricular/periventricular hemorrhage (Kuban et al., 1992). Randomized controlled trials have subsequently confirmed these results and a recent meta-analysis demonstrated that the frequency of CP or moderate/severe motor disability was reduced by ~30% by antenatal MgSO<sub>4</sub> (Doyle et al., 2009). Furthermore, the rate of echolucent and echodense brain lesions (Hirtz et al., 2015) as well as cerebellar hemorrhages (Gano et al., 2016) has been shown to be attenuated by antenatal MgSO<sub>4</sub> treatment.

Preconditioning is the induction of tolerance by a sub-lethal exposure to a subsequent severe insult (Meller and Simon 2013). A preconditioning response can be obtained in the immature brain by a variety of stimuli such as sub-threshold hypoxia (Gidday et al., 1994; Gustavsson et al., 2005) or xenon pre-exposure (Shu et al., 2010) which can reduce HI brain injury by > 80%. Most experimental studies have failed to confirm that MgSO<sub>4</sub> is neuroprotective if given immediately prior to, during or after HI (de Haan et al., 1997; Galvin and Oorschot 1998; Greenwood et al., 2000) but MgSO<sub>4</sub> administered to mothers in preterm labor is actually given hours to days before the presumed most critical period for development of brain injury. In a recent study (Koning et al., 2017), we demonstrated that MgSO<sub>4</sub> induces strong preconditioning when administered as a bolus 6 days to 12 h prior to the induction of HI in the PND 7 rat (which is developmentally similar to that of a human near-term fetus) (Vannucci and Vannucci 1997). But there is no information whether MgSO<sub>4</sub> also induces preconditioning in the more immature rodent brain better corresponding to extreme prematurity.

The aim of the present study is firstly to describe a reproducible, HI rat model (PND 4) of preterm brain injury more comparable to that seen in the extremely preterm (< 28 weeks of gestation) human infant. Secondly, we will evaluate the preconditioning effect of MgSO<sub>4</sub> in the

PND 4 rat model as well as in a previously described PND 5 mouse model (Albertsson et al., 2014) of preterm brain injury after HI.

## 2. Materials & methods

### 2.1. Experimental design

A power analysis was conducted prior to data acquisition in the different experiments contained in this study to make sure we would obtain an appropriate sample size for a reliable comparison. Throughout this study, pups were randomly allocated into different groups and numerically marked (as opposed to marked according to treatment) in order to avoid bias. Pups from a variety of litters were included in each experimental part to avoid litter specific outcomes and promote diversity. Additionally, all experiments of this study were conducted in replicates where the number and composition of replicates was determined by litter size, date of birth and amount of animals/samples possible to study at one time. Furthermore, all measurements were conducted by blinded researchers, unaware of the key to the numerical system by which the samples were marked. The data was then un-blinded at the time of statistical analysis.

Here we have used male and female Wistar rat pups from in house breeding at the facility of Experimental Biomedicine. Additionally, we used male and female wild type mice of the C57Bl/6 strain, also from in house breeding at Experimental Biomedicine. Animal experimental procedures conformed to guidelines established by the Swedish Board of Agriculture (SJVFS 2015: 38), were approved by the Gothenburg Animal Ethics Committee (ethical license 01-2016) and are reported in a manner consistent with the ARRIVE (Animal Research: Reporting in vivo Experiments) guidelines. Animals were housed at the facility of Experimental Biomedicine with access to food and water ad libitum. The day of birth was defined as PND 0. Mortality was low and not different between groups.

### 2.2. Hypoxia-Ischemia (HI)

At PND 4 (rats) or PND 5 (mice) pups were exposed to neonatal HI as previously described (Rice et al., 1981; Vannucci and Vannucci 1997) and adjusted to newborn mouse (Albertsson et al., 2014). Pups were anesthetized with 5% isoflurane (IsoFlo vet 100%; Abbott Laboratories Ltd, Illinois, USA) for induction and 1.5% for maintenance (< 5 min), in 1:1 oxygen-nitrogen mixture. The left common carotid artery was ligated with a 6.0 silk suture (Seide; Vömel, Germany) and the incision was closed and infiltrated with a local anesthetic (Xylocain 20 mg/ml, lidocain, hydrochloride; Astra Zeneca, Södertälje, Sweden). Pups were allowed to recover with their dams for 1 h before they were placed in a chamber with humidified air for 10 min, followed by 60, 70 or 80 min of hypoxia (8% oxygen in 92% nitrogen) for rats and 70 min for mice (10% oxygen in 90% nitrogen) and an additional 10 min in humidified air (temp 36 °C). Seven days post HI the pups were deeply anesthetized with 0.1 ml of thiopental (Pentocur, Thiopental, 50 mg/ml intraperitoneally (i.p.); Abcur AB, Helsingborg, Sweden) and sacrificed for histological processing; brains were fixed by transcardial perfusion using 6% paraformaldehyde (Histofix; Histolab, Gothenburg, Sweden).

### 2.3. MgSO<sub>4</sub> administration

Pups of either sex were divided randomly into two groups and injected intraperitoneally (i.p.) at PND 3 (rats) or PND 4 (mice) with a single dose of MgSO<sub>4</sub> (Magnesium, Addex; Fresenius Kabi, Halden, Norway) (Koning et al., 2017) or as vehicle an equivalent volume of saline (Saline 9 mg/ml, B Braun Melsungen AG, Melsungen, Germany) 24 h prior to the induction of HI (Table 1). Pups were then returned to their dams.

**Table 1**  
Summary of MgSO<sub>4</sub> doses used throughout this study.

Species	MgSO <sub>4</sub> dose	n
Rat	1.1 mg/g	18
Rat	Vehicle	18
Mouse	0.55 mg/g	20
Mouse	0.74 mg/g	14
Mouse	0.92 mg/g	19
Mouse	Vehicle	45

**Table 2**  
Brain injury scoring protocol for gross morphology modified after (Bona et al., 1998).

Score	Criteria
0	Normal
0.5	Almost completely normal, left hemisphere slightly smaller
1	Plaque on surface, minimal hypotrophy
1.5	Gradually more pronounced hypotrophy, with or without plaque
2	Obvious cavitation/loss of tissue
2.5	Cysts are added
3	Only midline left
3.5	Only frontal cortex left
4	Complete loss of left hemisphere

2.4. Macroscopical brain injury scoring in rats

Once dissected out the rat brains were photographed and graded macroscopically according to a previously published scoring system (range: 0–4), (Bona et al., 1998) see Table 2.

2.5. Tissue preparation

After dehydration, rat and mouse brains were embedded in paraffin and cut with a microtome (Meditome A550) into 7 µm thick sections at approximately –3.3 mm from bregma (level of hippocampus) as well as at approximately 0.2 mm from bregma (level of striatum) for histochemical staining as shown in Fig. 1.

2.6. Immunohistochemistry

Brain sections were prepared for immunohistochemical staining by deparaffination in xylene followed by graded alcohol rehydration and boiling in 0.01 M citric acid buffer (pH 6.0) for antigen recovery for 10 min. Sections were treated with 3% H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS) for blocking of endogenous peroxidase followed by blocking for nonspecific binding with appropriate serum. Sections were

then incubated with primary antibody: Mouse anti-MAP-2 (1:1000; M4403 Sigma-Aldrich, St. Louis, Missouri, USA) or Mouse anti-MBP (1:10 000; SMI-94 Covance, Princeton, New Jersey, USA) overnight followed by washing and incubation in appropriate secondary antibody and ABC Elite. For visualization of immunoreactivity, sections were submerged into 0.5 mg/ml 3,3-diaminobenzidine (DAB), NiSO<sub>4</sub>.

2.7. Thionin/Acid fuchsin

Histological analysis was undertaken using 7 µm sections at the levels of hippocampus and striatum. Oven baked sections were deparaffinated in xylene and rehydrated in graded alcohol and distilled H<sub>2</sub>O. For the assessment of gross morphological changes sections were stained with Thionine/Acid Fuchsin.

2.8. Brain injury analysis

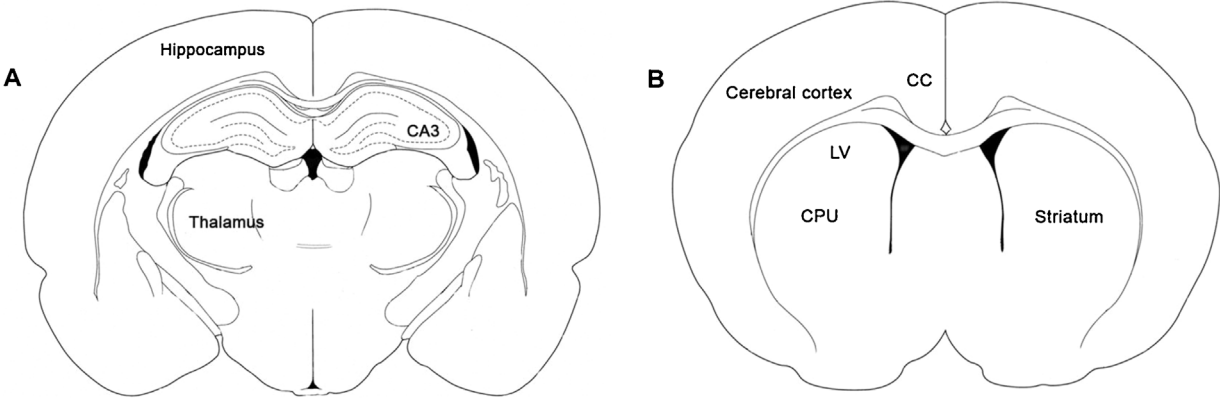
Grey matter injury was quantified as the area loss of microtubule-associated protein-2 (MAP-2) immunoreactivity at the levels of anterior hippocampus and striatum. The total area of MAP-2 positive staining in each hemisphere was outlined and measured (MicroImage, version 4.0, Olympus Optical) and the amount of tissue loss was calculated by subtracting the MAP-2 positive area in the ipsilateral hemisphere from the contralateral hemisphere.

Loss of myelin basic protein (MBP) was used immunohistochemically to determine white matter injury. The area of MBP positive staining in the corpus callosum was outlined and measured (MicroImage, version 4.0, Olympus Optical) in both hemispheres after which the proportion (%) of white matter injury was calculated by comparing the ipsilateral hemisphere to the contralateral hemisphere.

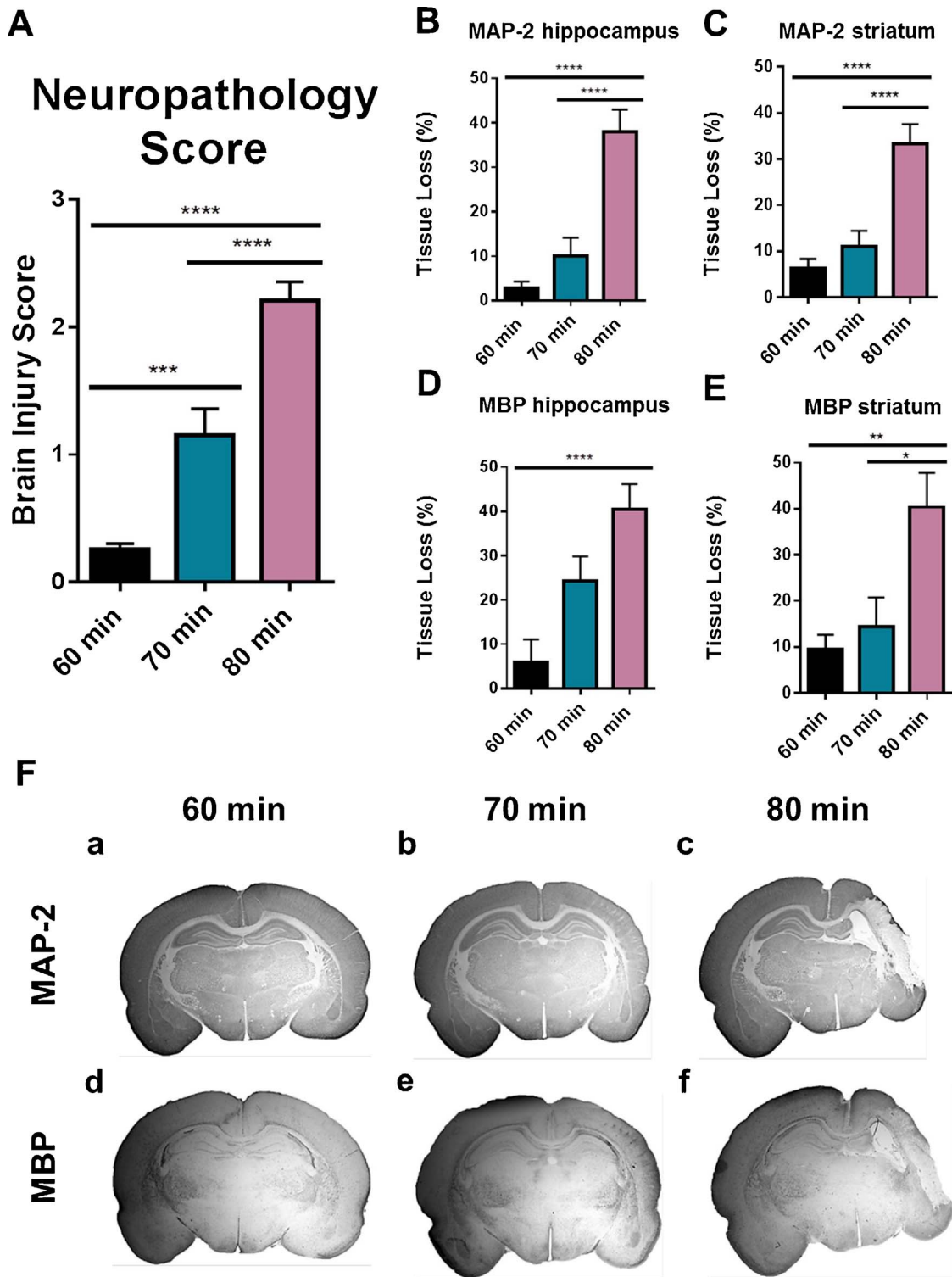
Changes in gross morphology and neuronal death were assessed by light microscopy of Thionine/Acid Fuchsin stained sections. The macroscopical injury was evaluated in the lateral ventricles and the microscopical injury was evaluated in cerebral cortex, hippocampus, striatum and thalamus.

2.9. Measurement of magnesium levels in serum

At PND 3 (rats) or PND 4 (mice) pups of either sex were injected *i.p.* with a single dose of MgSO<sub>4</sub> (rats: 1.1 mg/g, n = 19; mice: 0.55 mg/g (n = 12), 0.74 mg/g (n = 12) or 0.92 mg/g (n = 12) or an equivalent volume of saline as vehicle (rats: n = 20; mice: n = 14). Animals were killed by decapitation after 3 or 24 h followed by immediate collection of blood. The serum samples were then analyzed for Mg<sup>2+</sup> concentration (Cobas 8000 Roche Diagnostics Scandinavia AB) by Centrallaboratoriet Klinisk Kemi at the Sahlgrenska University Hospital,



**Fig. 1.** Schematic representation of the coronal sections of the rat brain analyzed in the present study. (A) Relevant anatomical structures at –3.3 mm from bregma depicted in this schematic image including Hippocampus, Thalamus and CA3 region. (B) Anatomical structures analyzed in sections at +0.2 mm from bregma including corpus callosum (CC), striatum, caudate putamen (CPU) and lateral ventricle (LV), (Adapted from the Rat Brain Atlas <http://labs.gaidi.ca/rat-brain-atlas/>).



**Fig. 2.** Evaluation of hypoxia duration in a PND 4 rat model of HI.

Brain injury evaluated 7 days post HI with (A) neuropathological scoring and immunoreactivity for MAP-2 (tissue area loss as % of contralateral) at the level of (B) hippocampus and (C) striatum and immunoreactivity for MBP (tissue area loss as % of contralateral) at the level of (D) hippocampus and (E) striatum. Representative coronal brain sections (F) stained with MAP-2 (a, b, c) and MBP (d, e, f) from a hypoxia duration of 60 min (a, d), 70 min (b, e) and 80 min (c, f) at the hippocampal level. One way ANOVA with Tukey's multiple comparisons test \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

Gothenburg, Sweden.

#### 2.10. Statistical measurements

All statistical analysis was performed using Graph Pad Prism. The statistical significance was determined by unpaired two-tailed  $t$ -tests or

ANOVA with Tukey's multiple comparisons test as described in the figure legends (Mean  $\pm$  SEM).  $P$  values  $< 0.05$  were considered statistically significant.



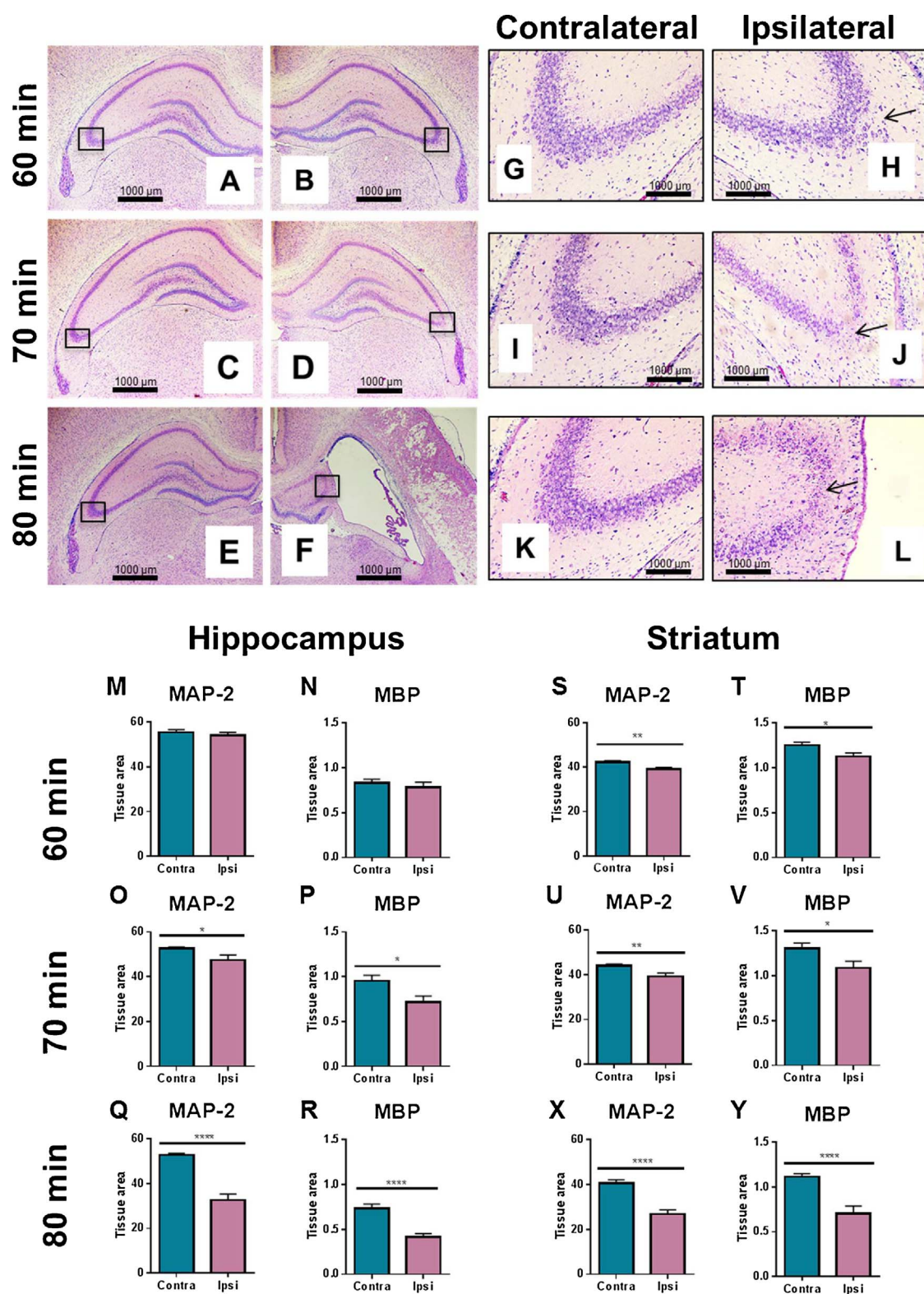


Fig. 3. Brain injury evaluated after 60, 70 or 80 min of HI in rats.

A–F. Representative thionin/acid fuchsin-stained sections of contralateral (A) and ipsilateral (B) hemisphere, 7 days after exposure to 60 (A & B), 70 (C & D) or 80 min (E & F) of HI (40× magnification). G–L. Representative images of thionin/acid fuchsin-stained sections in ipsi- and contralateral hemisphere of the hippocampal C3 layer after 60 (G & H), 70 (I & J) and 80 min (K & L), 400× magnification. Neuronal loss in this layer is indicated by arrows. M–R. Tissue area (mm<sup>2</sup>) in hippocampus of contralateral and ipsilateral hemisphere in grey and white matter 7 days after 60 (NS) (M & N), 70 (O & P) and 80 min (Q & R) of HI (n = 22/group), Mean ± SEM, Students unpaired *t*-test. S–Y. Tissue area (mm<sup>2</sup>) in striatum of contralateral and ipsilateral hemisphere in grey and white matter 7 days after 60 (S & T), 70 (U & V) and 80 min (X & Y) of HI (n = 22/group). Length indicator corresponds to 1000 μm. Mean ± SEM, Students unpaired *t*-test \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.0001.

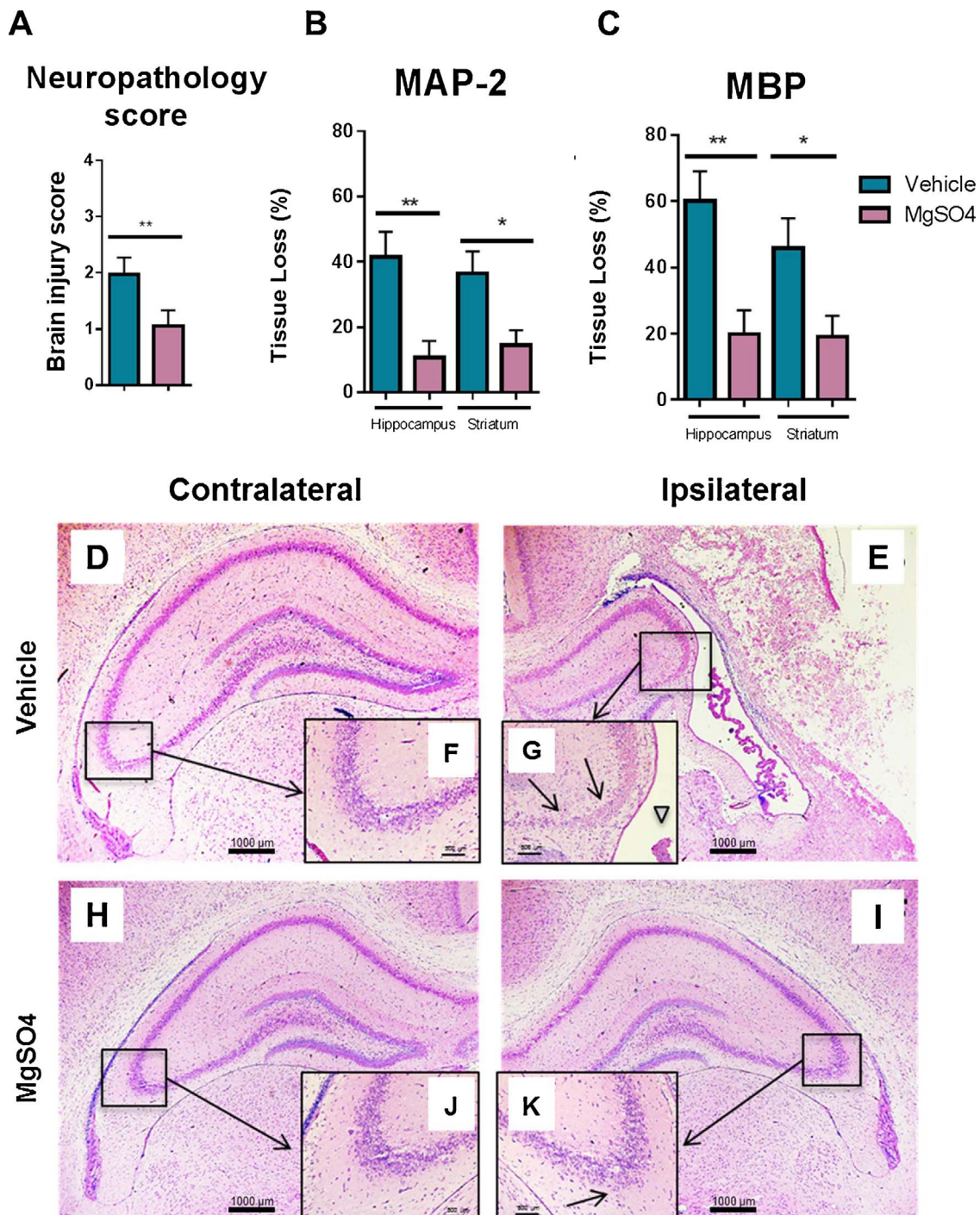


Fig. 4. Brain injury evaluation after MgSO<sub>4</sub> or vehicle pre-treatment and exposure to 80 min of HI in rats.

A–C. Brain injury evaluation with macroscopical scoring (A), and histochemical staining for MAP-2 (B) and MBP (C) 7 days post 80 min of HI in vehicle or MgSO<sub>4</sub> PC rat pups measured as tissue area loss (% of contralateral) at the level of hippocampus and striatum. D–E & F–G. Representative thionin/acid fuchsin-stained sections of hippocampus in the contralateral (D) and ipsilateral (E) hemisphere, 7 days after exposure to 80 min of HI in vehicle (D & E) or MgSO<sub>4</sub> (H & I) pre-treated pups, (4 × magnification). F–G & J–K. Pyramidal layer in the C3 region of hippocampus in the contralateral (F & G) and ipsilateral (K & J) hemisphere, (40 × magnifications). Neuronal loss indicated by arrows and vacuolation indicated by arrowhead. Length indicator corresponds to 1000 μm. Mean ± SEM, Students unpaired t-test \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.

### 3. Results

#### 3.1. Brain injury in white and grey matter in the PND 4 rat after HI

In order to determine the duration of hypoxia needed to produce a focal brain injury in both white and grey matter in the PND 4 rat we evaluated the injury obtained from left carotid artery ligation according to the Rice-Vannucci model followed by 60, 70 and 80 min of hypoxia.

Macroscopic scoring (Table 2, Fig. 2A) of brain injury showed that 60 min of hypoxia resulted in an injury in 50% of the animals though this injury was minor, consisting only of a slightly smaller ipsilateral hemisphere.

These results were subsequently confirmed by immunohistochemical staining for MAP-2 (Fig. 2B, 2C, 2F) and MBP (Fig. 2D–F) concluding that grey- (Fig. 3M) and white- (Fig. 3N) matter injury in the ipsilateral hemisphere was not significantly different after



60 min of hypoxia compared to the contralateral hemisphere at the level of hippocampus although a significant difference could be seen at the level of striatum in both grey- (Fig. 3S) ( $p < 0.01$ ) and white-matter (Fig. 3T) ( $p < 0.02$ ). Thionin/Acid Fuchsin staining revealed that after 60 min of hypoxia only 3/22 animals presented a hemisphere infarction in the cerebral hemisphere ipsilateral to the carotid artery ligation. The injury evaluation of cerebral cortex, hippocampus, striatum and thalamus showed very few and small isolated infarcts or groups of necrotic cells in the cerebral cortex (Fig. 3B, H), combined with mild or no atrophy at the level of hippocampus (Fig. 3B). The pyramidal layer of neurons in the C3 region of hippocampus was intact with minor neuronal loss (Fig. 3H). No injury was detected in the contralateral hemisphere after HI (Fig. 3A, G).

After 70 min of hypoxia, a higher percentage of animals (17/20) obtained a macroscopically visible brain injury (Fig. 2A) although the severity of the injury was inconsistent within the group and the pups showed only mild regional white matter injury (Fig. 2A, B, F) in the ipsilateral hemisphere as compared to the contralateral hemisphere (Fig. 2P) ( $p < 0.05$ ). Grey matter injury (Fig. 2A, 2B) could be detected in the cerebral cortex and hippocampus (Fig. 3D, 3J) of the ipsilateral hemisphere, characterized mainly by slight tissue area reduction in grey (Fig. 3O) ( $p < 0.03$ ) and white matter (Fig. 3P) ( $p < 0.02$ ) at the hippocampal level as well as at the level of striatum (Fig. 3U & V) ( $p < 0.01$  and  $p < 0.04$  respectively). A hemisphere infarction was detected in 3/20 brains. The ipsilateral hemisphere presented occasionally with small groups of necrotic cells detected in cerebral cortex, and degenerating neurons could be found in the hippocampal layer which also showed mild atrophy (Fig. 3D). When examining the C3 region of hippocampus we found moderate neuronal loss in the pyramidal layer and slight structural disruption (Fig. 3J). No such injury was detected in the contralateral hemisphere after HI (Fig. 3C, I).

80 min of hypoxia resulted in a consistent macroscopically detectable brain injury (Fig. 2) characterized by hippocampal atrophy and cortical infarction (Fig. 3F) in the ipsilateral hemisphere. All animals in this group obtained a macroscopically visible brain injury (Fig. 2A) and subsequent immunohistochemical staining with MAP-2 (Fig. 2B) and MBP (Fig. 2B) revealed extensive hippocampal and cortical injury. A significant reduction in both grey (Fig. 3Q) ( $p < 0.0001$ ) and white matter (Fig. 3R) ( $p < 0.0001$ ) was detected in the ipsilateral hemisphere compared to the contralateral hemisphere at the level of hippocampus. An equally severe injury was detected in grey (Fig. 3X) ( $p < 0.0001$ ) and white matter (Fig. 3Y) ( $p < 0.0001$ ) at the level of striatum.

After 80 min of hypoxia, 17/22 animals presented a hemisphere infarction (Fig. 3F) in combination with asymmetric lateral ventricles with moderate or extensive dilation in the ipsilateral hemisphere (Fig. 3F). The insult resulted in a moderate or extensive confluent infarct in the cerebral cortex accompanied by cell necrosis and infarction at the level of hippocampus (Fig. 3F). Extensive neuronal loss was detected in all animals in the pyramidal layer at C3 in the ipsilateral hemisphere after 80 min of hypoxia (Fig. 3L). No such injury was detected in the contralateral hemisphere after HI. Therefore 80 min of hypoxia was used for the remaining experiments in PND 4 rats.

### 3.2. Evaluation of brain injury in the PND 4 rat after $\text{MgSO}_4$ administration

Next, we sought to explore whether  $\text{MgSO}_4$  induces tolerance (preconditioning) in the preterm brain using our validated rat model of preterm brain injury. The macroscopic scoring of rat pups pretreated with  $\text{MgSO}_4$  or saline as vehicle at PND 3, 24 h prior to the induction of 80 min of HI, showed that there is a significant reduction of brain injury, from  $2.0 \pm 0.3$ ,  $n = 18$  in the vehicle group to  $0.9 \pm 0.3$ ,  $n = 18$  in the  $\text{MgSO}_4$  group ( $p = 0.0017$ ) (Fig. 4A).

For assessment of grey matter loss we measured the loss of immunoreactivity for MAP-2 in the ipsilateral brain hemisphere compared to the contralateral hemisphere, and found a significant brain injury

reduction of  $\sim 74\%$  in the  $\text{MgSO}_4$  pre-treated group at the level of hippocampus ( $p = 0.002$ ) and  $\sim 58\%$  reduction at the level of striatum ( $p \leq 0.01$ ), compared to vehicle (Fig. 4B).

Next, we assessed the effect of  $\text{MgSO}_4$  treatment on the loss of white matter by measuring myelin basic protein (MBP) immunoreactivity. We found a significant difference between rats pre-treated with  $\text{MgSO}_4$  vs vehicle with an injury reduction of  $\sim 67\%$  in the  $\text{MgSO}_4$  preconditioned group in hippocampus ( $p = 0.012$ ) and  $\sim 52\%$  in striatum ( $p = 0.02$ ) (Fig. 4C).

Using Thionine Acid/Fuchsin the extent of brain injury was scored both macroscopically and microscopically in paraffin sections. The brain injury in saline treated animals was characterized by macroscopically visible injury containing plaques and/or cysts in the ipsilateral hemisphere. The regional microscopic evaluation demonstrated that the majority of animals in this group suffered from infarction combined with moderate/extensive atrophy of cortex and hippocampus accompanied by cell necrosis in these areas combined with asymmetrical lateral ventricles dilated in the ipsilateral hemisphere (Fig. 4B). In the  $\text{MgSO}_4$  pre-treated group the macroscopically visible injury was less extensive and neuronal loss was limited (Fig. 4I). Furthermore, only a small proportion (2/20) of the brains exhibited an infarct after  $\text{MgSO}_4$  preconditioning as compared to (10/17) in the saline treated group. The amount of necrotic cells was reduced overall in the  $\text{MgSO}_4$  preconditioned group (Fig. 4F) and the pyramidal layer at C3 did not present the same extent of neuronal loss (degenerating neurons) as in the vehicle group (Fig. 4D vs. 4H).

Using immunoreactivity for MAP-2 and MBP, the total tissue area in grey and white matter was compared in the ipsilateral vs contralateral hemispheres of hippocampus in both the vehicle (saline) and the  $\text{MgSO}_4$  preconditioned group (Fig. 5A–D) after 80 min of HI. While a large reduction in tissue area of both grey and white matter was detected in the ipsilateral hemisphere in the vehicle group compared with the contralateral hemisphere ( $p < 0.05$ ), no significant grey or white matter tissue reduction in the ipsilateral hemisphere vs the contralateral hemisphere was detected in the  $\text{MgSO}_4$  pre-treated group. Analysis of grey matter tissue reduction at the level of striatum in the vehicle and the  $\text{MgSO}_4$  pre-treated group revealed a large loss of tissue in the vehicle group ( $p < 0.0001$ ) and a smaller but yet significant tissue loss in the  $\text{MgSO}_4$  group (Fig. 5C) ( $p < 0.02$ ). A significant tissue reduction was detected also in white matter in the ipsilateral hemisphere compared to the contralateral hemisphere at the level of striatum in the vehicle group ( $p < 0.0002$ ) whereas no significant tissue reduction was seen in the  $\text{MgSO}_4$  pre-treated group (Fig. 5D).

### 3.3. Effect of $\text{MgSO}_4$ on brain injury in the PND 5 mouse after HI

The effect of pre-treatment with  $\text{MgSO}_4$  was also analyzed in a PND 5 mouse model of preterm brain injury.  $\text{MgSO}_4$  was given 24 h prior to HI induced at PND 5 in mice using a newly characterized model of preterm brain injury (Albertsson et al., 2014). We found that a dose of 0.92 mg/g  $\text{MgSO}_4$  provided a significant reduction of grey matter injury (40%) compared to vehicle (Fig. 6A–C) also in this PND 5 mouse model, whereas the two lower  $\text{MgSO}_4$  doses (0.55 mg/g and 0.74 mg/g) (data not shown) did not provide any significant neuroprotection.

### 3.4. The magnesium levels in serum after $\text{MgSO}_4$ pre-treatment

The level of  $\text{Mg}^{2+}$  was measured in serum samples from rat pups preconditioned with 1.1 mg/g  $\text{MgSO}_4$  or saline as vehicle at 3 or 24 h prior to blood collection. In PND 4 rats, serum  $\text{Mg}^{2+}$  levels increase transiently (peaking at 3 h after administration) reaching  $4.1 \pm 0.2$  mmol/l after injection with 1.1 mg/g  $\text{MgSO}_4$ , and the  $\text{Mg}^{2+}$  levels were completely normalized at 24 h after injection (Fig. 6D). In the PND 5 mice, the neuroprotective dose of 0.92 mg/g  $\text{MgSO}_4$  reached a serum  $\text{Mg}^{2+}$  concentration of  $2.7 \pm 0.3$  mmol/l at 3 h after administration though the amount of  $\text{Mg}^{2+}$  was nearly but not completely



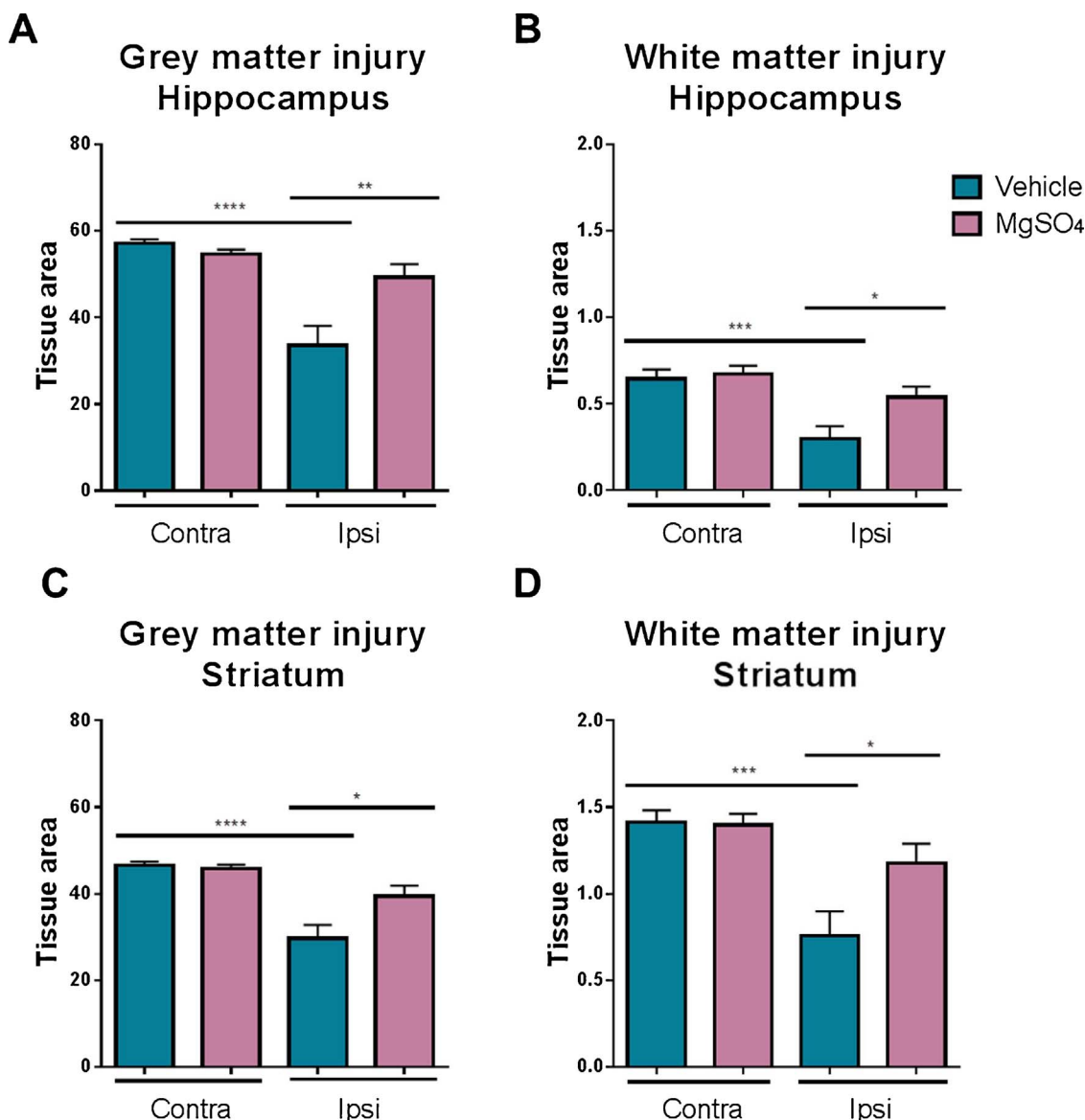


Fig. 5. Evaluation of grey and white matter tissue area reduction after MgSO<sub>4</sub> pre-treatment in PND 4 rats.

Total tissue area (mm<sup>2</sup>) at the levels of hippocampus and striatum between contralateral and ipsilateral hemisphere in grey (A & C) and white (B & D) matter, 7 days after 80 min of HI in vehicle (n = 19) and MgSO<sub>4</sub> (n = 19) pre-treated animals, Mean  $\pm$  SEM, Students unpaired *t*-test, \**p* < 0.05; \*\*\**p* < 0.001; \*\*\*\**p* < 0.0001.

normalized at 24 h (Fig. 6E) as in the PND 4 rat (*p* < 0.05).

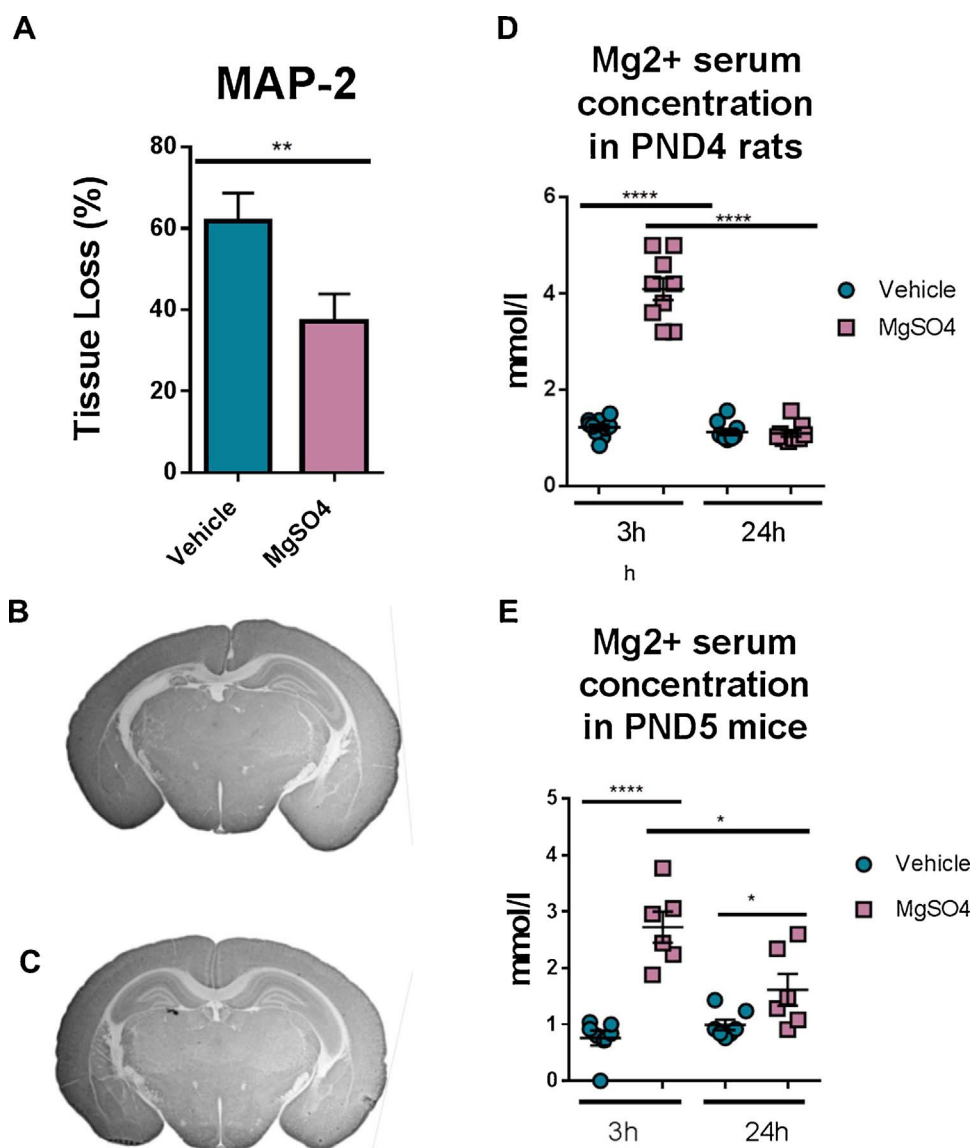
#### 4. Discussion

Here we have established a reproducible model of HI injury in the PND 4 rat for the study of perinatal brain injury in the very immature brain. Using this PND 4 rat model as well as an HI model in PND 5 mice, we found that MgSO<sub>4</sub> induces preconditioning neuroprotection in agreement with our previous findings in the PND 7 HI model in rats and ibotenate model in mice (Koning et al., 2017).

Comparing neural maturation between species is not simple but the developmental age of the PND 7 rat brain is generally considered to reflect that of a human fetus or newborn at 32–34 gestational weeks (GW) (Vannucci and Vannucci 1997) with complete cerebral cortical neuronal layering, little myelination of the white matter and involution of the germinal matrix (Towfighi et al., 1995). At the time of birth the neurogenesis of the rat brain is largely complete meaning that it developmentally corresponds to approximately 20 GW in the human (Clancy et al., 2001; Dobbing and Sands 1979). In order to mimic a more immature brain injury, corresponding to that of a ~28 GW

human fetus, we chose the PND 4 rat based on the white matter (Back et al., 2001) and cortical developmental stages (Honig et al., 1996).

After the duration of 70 min of hypoxia, 85% of the rat pups obtained a macroscopically visible injury extending through both white and grey matter, characterized by mild atrophy and very limited neuronal loss in hippocampus accompanied by small groups of degenerating neurons in the cerebral cortex. A mild infarct in cortex or hippocampus was seen in ~13% of the animals. HI for 80 min induced injury in all rat pups, largely restricted to the cerebral hemisphere ipsilateral to the carotid artery ligation. The injury was characterized by moderate hippocampal atrophy and subcortical/periventricular white matter damage in cerebral cortex which is different from that seen after injury at PND 7. The higher proportion of white matter injury also better corresponds to brain injury in preterm infants (Inder et al., 1999b; Pierson et al., 2007). In 2003 Sizonenko et al. adapted the Rice-Vannucci model to PND 3 rats aiming to mimic the injury of a human fetus at 25–28 GW. The setup of our model differs slightly from that used by Sizonenko et al. partially because they place the pups in 85–90% humidity for 30 min after ligation, directly followed by 30 min 6% hypoxia whereas we allow the pups 1 h rest with the dam followed



**Fig. 6.** Brain injury evaluation after MgSO<sub>4</sub> or vehicle pre-treatment and exposure to 70 min of HI in PND 5 mice and serum Mg<sup>2+</sup> concentration.

A. Brain injury measured as ipsilateral tissue area loss (% of contralateral) in grey matter by immunoreactivity for MAP-2 at the level of hippocampus in PND 5 mice after pre-exposure to MgSO<sub>4</sub> (0.92 mg/g) or an equivalent volume of saline as vehicle. (Mean  $\pm$  SEM, Students unpaired t-test,  $p < 0.05$ ). B–C. Representative brain sections from mice pre-treated with vehicle (B) or MgSO<sub>4</sub> (C) at the level of hippocampus with MAP-2 staining. D. Serum Mg<sup>2+</sup> concentration measured in PND 4 rat samples collected at 3 or 24 h after i.p. injection with MgSO<sub>4</sub> (1.1 mg/g) ( $n = 9$ ) or vehicle ( $n = 9$ ). E. Serum Mg<sup>2+</sup> concentration measured in PND 5 mice samples collected at 3 or 24 h after i.p. injection with MgSO<sub>4</sub> (0.92 mg/g) ( $n = 7$ ) or vehicle ( $n = 6$ ), Mean  $\pm$  SEM, Students unpaired t-test, \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

by 80 min 8% hypoxia. Additionally, they detect a difference for the ipsi- and contralateral hemispheres after HI compared to control only in the parietal cortex (Sizonenko et al., 2003). Hence there is no significant difference between the ipsi- and contralateral hemispheres in the thalamus, hippocampus or striatum after HI in their model whereas we detect significant brain injury in cortex as well as in hippocampus and striatum in our model.

MgSO<sub>4</sub> (1.1 mg/g) preconditioning has neuroprotective properties with a brain injury reduction of ~74% in grey matter and ~64% in white matter at the level of hippocampus in this PND 4 rat model of HI which is comparable to the injury reduction seen by MgSO<sub>4</sub> given 24 h prior to HI in the PND 7 rat (~63% in grey matter and ~89% in white matter, Koning et al., 2017). Preconditioning with MgSO<sub>4</sub> reduced the amount of cellular necrosis and protected the C3 hippocampal layer from extensive neuronal loss. Additionally, the cerebral cortex was largely protected against the vast tissue and neuronal loss seen in the control group.

We also found that the MgSO<sub>4</sub> neuroprotection seen in this PND 4 rat model can be obtained in a PND 5 mouse model of perinatal brain injury. Additionally, the serum Mg<sup>2+</sup> concentrations measured in both models 3 h post injection were within the therapeutic window (2–4 mmol/L) when MgSO<sub>4</sub> is given antenatally to women for seizure prophylaxis in preeclampsia or for neuroprotection. Taken together

with our recently published data (Koning et al., 2017), we have now demonstrated that MgSO<sub>4</sub> provides preconditioning neuroprotection in four brain injury models (HI at PND 4 rat, PND 7 rat and PND 5 mouse as well as mouse PND 5 ibotenate lesion), supporting that this phenomenon is generalizable to some extent irrespective of species, type of insult and developmental age.

The present results together with our previous report offers some mechanistic insights into how MgSO<sub>4</sub> protects the immature brain, i.e. it has to be given as a pre-exposure at least  $\geq 12$  h prior to the insult (Koning et al., 2017) whereas MgSO<sub>4</sub> is often not effective if given just prior, during or after the insult (de Haan et al., 1997; Galvin and Oorschot 1998; Greenwood et al., 2000; Koning et al., 2017). We believe that MgSO<sub>4</sub> offers preconditioning, i.e. it reduces the vulnerability of the immature brain similar to subthreshold hypoxia (Gidday et al., 1994; Gustavsson et al., 2005; Meller and Simon 2013), glucocorticoids (Barks et al., 1991) or xenon (Gustavsson et al., 2005; Shu et al., 2010). Such an interpretation is supported by the fact that the serum magnesium concentrations are normalized (or nearly normalized) at the time of the severe insult and still brain injury is decreased dramatically. MgSO<sub>4</sub> changes the expression of a number of genes which in turn alters the mitochondrial and metabolic state of the immature CNS which renders it more resistant to a subsequent severe insult (Koning et al., 2017). However, this genomic/metabolic transformation into a more

resistant state develops over time and is not complete until 12–24 h although it seems to last for over 6 days, at least in the PND 7 rat model.

Clinically, it is of importance that this preconditioning neuroprotection induced by a  $\text{MgSO}_4$  bolus has now also been shown in two preterm models of HI. There is a need for development of preventive approaches and the data presented in this study support previous clinical findings demonstrating that  $\text{MgSO}_4$  is an effective neuroprophylaxis when administered antenatally. That is, treatment must start some time before the actual critical period for insults to the brain during delivery and the first few days of life. The possibility of offering only a  $\text{MgSO}_4$  bolus rather than also giving infusions of  $\text{MgSO}_4$  during delivery up until the time of birth reduces the need for extensive monitoring of the mother and could also possibly decrease the risk of necrotizing colitis, gut perforations and perinatal mortality (Borja-Del-Rosario et al., 2014; Kamyar et al., 2016; Ratray et al., 2014). Indeed, in one of the randomized controlled studies (Marret et al., 2007) only a bolus of 4 g  $\text{MgSO}_4$  was given. The long-term effects were promising (Chollat et al., 2014) but, unfortunately, the study was underpowered. We further believe that 6 g (rather than 4 g) of  $\text{MgSO}_4$  is needed to reach a transient peak of magnesium in serum of 2.5–4 mmol/l which is required to achieve optimal brain protection, at least judged from our experimental studies. Clinical studies are warranted to examine if a 6 g  $\text{MgSO}_4$  bolus is an effective mode of neuroprophylaxis with greater safety and compliance for women in preterm labor.

### Contributor's statements

GK participated in the design of the study, performed the animal experiments, carried out the analyses, performed the statistical analysis and drafted the initial manuscript. EL assisted in some of the experimental work and performed some of the tissue preparations.

PS assisted in some of the animal experiments and performed part of the tissue preparations. A-LL assisted in some of the experimental work and performed some of the tissue preparations. MJ assisted in some of the experimental work and performed some of the tissue preparations. PG participated in data interpretation. CT participated in data interpretation and offered professional help with manuscript writing. XW participated in data interpretation. CM took part in designing the study and participated in data interpretation. HH conceptualized and designed the study, obtained funding, interpreted data and took active part in writing the manuscript. All authors reviewed and revised the manuscript and approved the final manuscript as submitted.

### Conflicts of interest

The authors declare no conflicts of interest.

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